Could multiple low-affinity bonds mediate primary sperm–zona pellucida binding?

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The current model for primary sperm binding to the zona pellucida is that a cell-surface sperm protein binds with high affinity in an order-specific manner to one of the zona pellucida proteins, ZP3. However, the molecular details of primary sperm–zona pellucida binding remain elusive. A possible revised model is that multiple low-affinity bonds between spermatozoa and the zona pellucida may be sufficient for primary binding. The avidity of several low-affinity bonds can exceed that of a single high-affinity bond, which is sufficient to tether a motile spermatozoon. A mechanism involving multiple low-affinity bonds could account for some of the difficulties in elucidating primary sperm–zona pellucida interactions.

In the early 1980s, Wassarman *et al.* published a series of seminal biochemical investigations on the mouse zona pellucida (Bleil and Wassarman, 1980; Bleil *et al.*, 1981; Greve and Wassarman, 1985), the extracellular glycoprotein matrix that surrounds the mammalian egg, in which its protein constituents, ZP1, ZP2 and ZP3, were identified and named on the basis of molecular mobility on SDS-PAGE gels. Furthermore, a model was developed for sperm–zona pellucida interactions. Capacitated spermatozoa, after reaching the oviduct, bind to the zona pellucida of ovulated eggs via an order-specific, high-affinity interaction between a sperm outer membrane protein and mouse ZP3. Binding triggers a sperm exocytotic event, the acrosome reaction, which leads to secondary binding between a sperm acrosomal membrane protein and mouse ZP2, and penetration of the zona pellucida. Mouse ZP1 is not involved directly in sperm–zona pellucida interactions but serves as a cross-linking protein for stability of the zona pellucida matrix. Subsequent studies demonstrated that monoclonal antibodies to mouse ZP2 and ZP3 could block fertilization *in vivo*, which strengthened the case for the functions attributed to these proteins (East *et al.*, 1984, 1985).

A number of sperm proteins have been identified as candidates for primary binding to the zona pellucida. However, experimental genetic 'knock-out' or null models of these candidates in mice have failed to provide convincing evidence for any of these proteins as the primary zona-binding protein. For example, mice null for $\beta1,4$-galactosyltransferase (GalTase) have markedly reduced fertility, but fertility is not completely eliminated (Lu and Shur, 1997); the broad spectrum of negative physiological effects, including pituitary insufficiency and neonatal lethality, raises doubts as to whether the effects on fertility are direct or indirect (Lu *et al.*, 1997). Genetic manipula-

tions of mouse ZP3 have also been difficult to interpret. Mice null for ZP3 do not have a zona pellucida (Liu *et al.*, 1996; Rankin *et al.*, 1996). Genetic introduction of human ZP3 to mice null for mouse ZP3 restored the zona pellucida (chimaeric zona pellucida composed of human ZP3, mouse ZP3 and mouse ZP1) but did not change the phenotype of the mouse zona pellucida in that the chimaeric zona pellucida, like the wild-type mouse zona pellucida, bound mouse but not human spermatozoa (Rankin *et al.*, 1998). The authors presented several hypotheses to explain this phenotype: (1) post-translational modification of human ZP3 with mouse-specific biological activity; (2) subtle alterations to the supramolecular structure of human ZP3 that favour mouse sperm binding; and (3) the requirement for a second human zona pellucida component for human sperm binding.

In light of these genetic experiments, an alternative mechanism for primary sperm–zona pellucida binding is proposed: sperm initially bind to the zona pellucida via multiple low-affinity bonds in the absence of a high-affinity receptor–ligand interaction. In the same way that decavalent immunoglobulin M (IgM) can achieve avidity that approaches or exceeds that of bivalent IgG (Crothers and Metzger, 1972; Hornick and Karush, 1972; Roitt *et al.*, 2001), the requisite avidity for binding between spermatozoa and the zona pellucida could be achieved via low-affinity bonds. Baltz *et al.* (1988) demonstrated that one or a few non-covalent bonds with an affinity of a typical antibody–antigen interaction ($K_A \approx 10^6–10^{10}$ M^{-1}) were sufficient to 'tether' a spermatozoon. Upon binding, there is a large contact area between the spermatozoon and the zona pellucida as the spermatozoon appears to be bound not at the tip of the sperm head but rather at the flattened peri-equatorial region of the head; it is of note that antibodies to several antigens found in this region of the sperm head block sperm binding (Primakoff *et al.*, 1985)

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Table 1. Primary sperm–zona pellucida interactions between animal species

Zona pellucida	Spermatozoa																
	Human	Gibbon	Baboon	Rhesus monkey	Squirrel monkey	Marmoset	Cynomolgus monkey	Boar	Ram	Cow	Horse	Pig	Rabbit	Rat	Guinea-pig	Hamster	Mouse
Human	+	+				+	−				+		+			+	+
Gibbon	+																
Baboon	−		+										+			+	+
Rhesus monkey	−			+	+		+						+				
Squirrel monkey	−			+	+								+		+		
Marmoset	−				+	+											
Cynomolgus monkey							+										
Boar								+				+					
Ram									+	+							
Cow									+	+							
Horse	−										+	+					
Pig	−							+				+	+			+	+
Rabbit	−												+	+	+, +/−	+	+
Rat														+		+	
Guinea-pig	−												+		+	−	
Hamster	−												+		−	+	+
Mouse	−												+		−, +/−	+	+

+: positive binding; +/−: weak binding; −: no binding.

Data from Bedford, 1977; Russell *et al.*, 1980; Schmell and Gulyas, 1980; Swenson and Dunbar, 1982; Uto *et al.*, 1988; Slavik *et al.*, 1990; Liu *et al.*, 1991; VandeVoort *et al.*, 1992; Oehninger *et al.*, 1993.

and fertility (Saling *et al.*, 1983; Naz *et al.*, 1984; Primakoff *et al.*, 1988). Many low-affinity bonds could form a sufficiently strong bond, equivalent to a few high-affinity bonds needed for binding (Baltz and Cardullo, 1989), to tether spermatozoa temporarily, but could then yield to secondary interactions resulting in penetration of the zona pellucida by spermatozoa. If enough bonds formed rapidly, the torque developed from sperm motility would be unlikely to break such an interaction.

Is such a mechanism plausible? Given the logarithmic relationship between bond energy (strength) and affinity, several 10^4 M^{-1} 'weak' bonds will surpass the bond strength of a single 10^{10} M^{-1} bond. A typical affinity bond is nothing more than a composite of weak interactions, such as ionic bonds, hydrogen bonds, Van der Waals interactions and hydrophobic interactions. In the extreme, these weak interactions need not be limited to a binding pocket, as envisioned by classical biochemistry, but could be spread across the interface between the spermatozoon and the zona pellucida. A requirement is that this mechanism must be sufficient to trigger the sperm acrosome reaction, which presumably is required for sperm penetration of the zona pellucida. Alternatively, a second high-affinity interaction could be responsible for triggering the acrosome reaction.

If the single components in the interaction are low affinity, they are unlikely to be specific and, contrary to current views, it might be expected that primary sperm–zona pellucida interactions are not order-specific. Indeed, cumulative evidence indicates that primary sperm–zona pellucida binding may not be highly order-specific (Table 1 and references therein), possibly with the exception of human spermatozoa, which appear to bind only to human and gibbon zonae pellucidae. The specificity of human spermatozoa appears to be the exception, not the rule, and may be the result of unique evolutionary pressures. For example, the environment of the human vagina is uniquely acidic and has a typical pH range of 3.5–4.5 (Boskey *et al.*, 1999) compared with values of 5.9–7.0 (Johnson *et al.*, 1984; Miller, 1994), 7.0 (Lichtenwalner *et al.*, 2000), 5.5–6.0 (Johnson *et al.*, 1984) and 7.0 (Johnson *et al.*, 1984) for pig-tail macaques, rhesus macaques, chimpanzees and tamarins, respectively. Thus, to promote fertility, human spermatozoa may have adapted, and these changes, possibly to the sperm outer membrane, may inhibit interactions with heterologous zonae pellucidae. Unlike human spermatozoa, the human zona pellucida appears to be permissive to primary binding by heterologous spermatozoa.

In vitro zona-binding assays have been used to measure the inhibition of sperm binding by specific biochemical moieties. For example, Thaler and Cardullo (1996) and Johnston *et al.* (1998) demonstrated the inhibition of sperm binding by the addition of specific sugar moieties. It is noteworthy that 100% inhibition of binding was not achieved in either study. Moreover, the authors in each of these studies proposed specific sperm proteins involved in primary sperm–zona pellucida binding that either have not yet been proven to be important (fucosyltransferase) or may not even be found on the outer membrane (SP56; Foster *et al.*, 1997). Neither study could differentiate between high-affinity bonds and multiple low-affinity bonds creating an equivalent avid bond. Thus, although both low- and high-affinity bonds may participate in binding, as suggested in both studies, high-affinity bonds may not be an absolute requirement for primary binding leading to fertilization. As suggested above, a second high-affinity sperm–zona pellucida interaction may be necessary for acrosomal activation.

Conclusion

The absence of confirmatory genetic data does not preclude the presence of one or several zona-binding proteins on the sperm outer membrane that bind with high affinity and specificity to ZP3. Indeed, a recent study has identified new sperm proteins that could invalidate a low-affinity, high-avidity model, but the role of these proteins awaits further experimental confirmation (Thaler and Cardullo, 2002). However, these data also do not preclude an additional molecular mechanism of multiple low-affinity bonds that create sufficient avidity for sperm–zona pellucida binding and triggering of the acrosome reaction. It is possible that both mechanisms operate (Thaler and Cardullo, 1996; Johnston *et al.*, 1998) and that the mechanism involving low-affinity bonds serves as a safety mechanism to ensure fertility. An example is the persistence of fertility in mice that are null for GalTase. It is possible that the ability of spermatozoa to undergo the acrosome reaction spontaneously is a further safeguard for fertilization. It is remarkable that the molecular details of primary binding remain elusive.

As a proposed alternative, this low-affinity, high-avidity mechanism is problematic because it is difficult to validate experimentally. Re-creating the molecular events with the correct molecular constituents involved in forming a single low-affinity bond is inherently challenging using standard molecular techniques. Should these moieties be identified, it is predicted that their ability to inhibit primary sperm–zona pellucida binding will be greatly enhanced by being displayed in two- and three-dimensional arrays that approximate the surfaces of the spermatozoon and the zona pellucida. A second prediction is that genetic knock-out of candidate sperm proteins will fail to eliminate completely primary binding, except in cases in which broad physiological changes occur as a consequence.

Results of recent genetic analyses have suggested that ZP2 and ZP3 evolve rapidly, which could explain the order specificity of sperm–zona pellucida interactions (Swanson *et al.*, 2001). However, as mentioned above, primary binding does not appear to be highly order-specific. Furthermore, such a hypothesis would also indicate a parallel co-evolution of sperm proteins that would be required to maintain sperm–zona pellucida binding, which, to date, has not been identified. A less specific mechanism may be all that is required for primary sperm–zona pellucida binding,

given the already substantial barriers to cross-fertilization between animal orders (O'Rand, 1988).

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